

From: Thea Sesardic
Sent: Friday, March 10, 2006 1:44 PM
To: NIEHS NICEATM
Subject: Botulinum toxin - Alternative methods

Dear Dr William S Stokes,

I have sent today by currier delivery information related to proposed workshop on Alternative Methods to Refine, Reduce and Replace the Mouse LD50 Assay for Botulinum Toxin Potency Testing.

After working on this topic for nearly 15 years I am happy to be able to share experience with NTP/NICEATM and ICCVAM in view of eliminating LD50 test.

SOP's for methods used at NIBSC are included as pdf files in this Email together with short summary of information that was sent by currier. Copies of all published documents together with example of data was included in delivery package. It is not possible to provide raw data and details of all assays we have accumulated over the last 15 years - however we have provided examples of what we can do and further discuss on workshop. Please do not copy the data provided unless we agree on how it will be used.

After review of data you could suggest to focus on a particular assay for which we than could provide more information if required.

Kind regards and I look forward to hearing from you.

Thea Sesardic

Dorothea (Thea) Sesardic PhD
Principal Scientist
Division of Bacteriology
NIBSC
Hertfordshire, UK

Response to

**Department of Health and Human Services
National Institute of Health**

**NTP Interagency Center for the Evaluation of Alternative Toxicological
Methods (NICEAM)**

**Nomination to hold Workshop on Alternative Methods to Replace the Mouse
LD50 Assay for Botulinum Toxin Potency Testing**

Experts:

Dr Dorothea (Thea) Sesardic

Principal Scientist
Division of Bacteriology
National Institute for Biological Standards and Control (NIBSC)
Hertfordshire, UK

Position: Group leader, bacterial toxin laboratory with >15 years experience on testing of therapeutic formulations of Botulinum toxins.

Dr Rose Gaines Das

Head of Biostatistics
NIBSC

Position: Head of Biostatistics with >25 years experience on validation of assay methods

Dr Russell G A Jones

Senior Scientist
Division of Bacteriology
NIBSC

Position: Expert on botulinum toxins and botulinum anti-toxins with 5 years experience.

List of publication on assay methods for reduction, refinement and replacement for potency assays of Botulinum toxins in support of nomination for NICEATM

In vivo LD50 (reduction/standardisation)

McLellan K, Gaines Das R, Ekong TAN and Sesardic D. (1996). Therapeutic botulinum type A toxin: factors affecting potency. *Toxicon*, **34**, 975-985.

Sesardic D, Gaines Das RE and Corbel MJ (1994). Botulinum toxin: How to define biological activity. *Journal of the Royal Society of Medicine (letter)* **87**, 307.

Sesardic D, Leung T, Gaines Das R (2003). Role of standards in assays of botulinum toxins: international collaborative study of three preparations of botulinum type A toxin. *Biologicals*, 31: 265-276.

Jones RGS, Corbel MJ and Sesardic D. (2006). A review of WHO International Standards for Botulinum Antitoxins. *Biologicals*, (in press).

In vivo non-LD50 (refinement)

Sesardic D, McLellan K, Ekong TAN and Gaines Das R. (1996). Refinement and validation of an alternative bioassay for potency testing of therapeutic botulinum type A toxin. *Pharmacology & Toxicology* 78, 283 - 288.

Sesardic D, Jones RGA, Leung T, Alsop T, Tirney R. (2004) Detection of antibodies against botulinum toxins. *Movement Disorders*, 19: 85-91.

In vitro (replacement)

Ekong TAN, McLellan K and Sesardic D. (1995). Immunological detection of *Clostridium botulinum* toxin type A in therapeutic preparations. *Journal of Immunological Methods* **180**, 181-191.

Ekong TAN, Feavers I and Sesardic D. (1997). Recombinant SNAP-25 is an effective substrate for *Clostridium botulinum* type A endopeptidase activity. *Microbiology*, **143**, 3337-3347.

Ekong TAN, Gee C, Blasi J and Sesardic D. (1997). An alternative bioassay for botulinum neurotoxin type A based on its endopeptidase activity. In *Animal Alternatives, Welfare and Ethics* (Eds: LFM van Zutphen and Balls M). *Developments in Animal and veterinary Sciences* 27, 1039-1044.

Sesardic D, Corran PH, McLellan K, Feavers I and Ekong TAN (1997). *In vitro* assays for estimating the activity of therapeutic preparations of botulinum toxin. In: *Animal Alternatives, Welfare and Ethics* (Eds: LFM van Zutphen and Balls M). *Developments in Animal and Veterinary Sciences* 27, 1033-1038.

Gaines-Das RE, Heath AB, Martin H and Sesardic D. (1999). Validation of in vitro assays for Botulinum Toxin: A case study. In *Alternatives to Animals in the*

Development and Control of Biological Products for Human and Veterinary Use. (Eds Brown F, Hendriksen C and Sesardic D). *Developments in Biological Standardization* Vol 101. pp267-276.

Sesardic D, Corran P, Gee C and Ekong TAN. (2000). *In vitro* approaches for estimating activity of tetanus toxin as an alternative assay for specific toxicity. In: “*Progress in the reduction refinement and replacement of animal experimentation*” (Balls M, van Zeller A-M and Halder M Eds.), pp 969-974.

Sesardic D, Martin H, Tierney R and Bigalke H. (2000). An *in vitro* assay for testing of neutralising antibodies to botulinum toxins. In: “*Progress in the reduction refinement and replacement of animal experimentation*” (Balls M, van Zeller A-M and Halder M Eds.), pp 1001-1008.

General : reviews and related relevant publications

Corran PH and Sesardic D. (1994). Meeting Report: International Workshop On Bacterial Toxins in Medical Use. *Biologicals* **22**, 83-84.

Sesardic D. (1996). Requirements for valid alternative assays for testing of biological therapeutic agents. *Developments in Biological Standardization* **86**, 311-318.

Brown F, Hendriksen CFM and Sesardic D (Eds). (1999) Alternatives to Animals in the Development and Control of Biological Products for Human and Veterinary Use. *Developments in Biological Standardization* Vol 101.

Sesardic D. (1999). Alternatives to the use of animals for bacterial toxins and antitoxins. In : Celebration of 50 years of progress in biological standardization and control at WHO. *Developments in Biological Standardization*, 100:75-82.

Brown F, Hendriksen CFM, Cussler C and Sesardic D. (Eds). (2002). Advancing Science and Elimination of the Use of Laboratory Animals for Development and Control of Vaccines and Hormones. *Developments in Biological Standardization* Vol 111, Karger Press, Basel. Switzerland.

Leung T, Corran P, Gee C, Ekong TAN and Sesardic D. (2002). Application of an *in vitro* endopeptidase assay for detection of residual toxin activity in tetanus toxoids. In Brown F, Hendriksen CFM, Sesardic D & Cussler K (Eds). Advancing Science and Elimination of the Use of Laboratory Animals for Development and Control of Vaccines and Hormones. *Developments in Biological Standardisation*, 111: 335-340.

Meunier FA, Lisk G, Sesardic D and Dolly OJ. (2003). Dynamics of motor nerve terminal remodeling unveiled using SNARE-cleaving botulinum toxins: the extent and duration are dictated by the sites of SNAP-25 truncation. *Molecular and Cellular Neuroscience*, 22: 454-466.

European Pharmacopoeia Monograph

Botulinum Toxin Type A for injection. European Pharmacopoeia 01/2005:2113.

Standard operating procedures

1. Non-lethal mouse local muscular paralysis assay: In vivo assessment of botulinum type A toxin
2. Phrenic nerve hemidiaphragm assay: *In vitro (ex vivo)* assessment of botulinum neurotoxicity.
3. *In vitro* SNAP-25 endopeptidase immunoassay for potency testing of botulinum toxin A preparations.

Data to be presented at the workshop or included in surface mail:

1. **Mouse LD50:** method as in relevant publications. This test was performed at NIBSC from 1991-1996 after which it was replaced by in vivo mouse paralysis test. It is no longer performed at NIBSC for potency testing of therapeutic products since 1996. LD50 data used in comparisons with in vitro SNAP-25 assay were generated by marketing authorisation holders and therefore confidential. Collaborative study compared LD50 assays in 10 laboratories (published data).
2. **Mouse paralysis:** method as in published literature and in electronic version of SOP. Test performed routinely for type A botulinum toxin at NIBSC from 1996-2000 (>10 assays per year) and thereof only for annual re-calibration of product specific reference standards for use in vitro batch release test. Example data with statistical evaluation provided from one recent assay and described in memo from Rose E Gaines Das to D Sesardic 01 March 2006 to be sent in package by surface mail. In house experience also with type B therapeutic toxin. Examples of calibration of product specific reference – confidential information.
3. **Mouse isolated phrenic nerve hemidiaphragm:** method as in electronic version of SOP. At present under in house validation for confirming potency of bulk active toxin and product specific reference standards. Limited and only unpublished data. Example of dose response curve for type A toxin on poster presented on the 5th International Conference on Basic and Therapeutic Aspects of Botulinum and Tetanus Toxins, Botulinum, Denver Colorado, USA June 2005.
4. **SNAP-25:** method as in relevant literature and electronic version of SOP. In routine use at NIBSC since 1999 for batch release of type A botulinum toxin products. Data compared with LD50 using MAH LD50 data. Examples provided for information taking out actual lot numbers but should not be copied without prior consent.
5. **Rat primary spinal cord cell:** preliminary unpublished data on dose response of inhibition of 3H glycine release following incubation with botulinum toxin A.

**NATIONAL INSTITUTE FOR BIOLOGICAL STANDARDS AND
CONTROL**

Division of Bacteriology

Standard Operating Procedure

Phrenic Nerve Hemidiaphragm Assay

***In vitro (ex vivo)* assessment of botulinum neurotoxicity**

Written by: Dr R G A Jones

Authorized by: Dr D Sesardic

Introduction

Botulinum toxin type A induces muscular paralysis following specific binding and uptake by the pre-synaptic nerve terminal, and subsequent cleavage of SNAP25, a protein essential for the release of neurotransmitter at the synaptic junction. There are three separate functional regions of the toxin responsible for specific binding, translocation and enzymatic activities respectively. Loss of any one of these activities will result in an inactive toxin molecule, as assessed by a fully functional model such as the mouse LD₅₀ test. Following the three R's principle, alternative fully functional assays are required. Use of the *ex-vivo* phrenic nerve hemidiaphragm assay therefore, full-fills this criteria.

Electrical stimulation of the phrenic nerve causes twitch responses in the focally innervated mouse diaphragm muscle. The diaphragm muscle consists of a thin sheet which remains viable for hours in an organ bath, allowing the inhibitory effects of botulinum neurotoxin to be directly measured. Toxin potency is directly measured by the degree of muscular paralysis induced by the toxin acting on the pre-synaptic nerve terminal. Activity of a toxin batch is estimated relative to a product specific toxin reference material of defined activity.

Materials / Methods

Equipment

Computer: Dell Optiplex with 1.25GB RAM

PowerLab/4SP 4 channel recorder (ADInstruments, UK)

Bridge Amp ML110 (ADInstruments, UK)

4 Channel Dual Impedance Stimulators (ADI, Digitimer, UK)

Isometric force transducers, GM 2 type (FMI, Germany)

Hemidiaphragm Equipment / tissue baths / holders (FMI, Germany)

Thermocirculator (Harvard Apparatus, UK)

95% Oxygen/5% Carbon dioxide Gas cylinder (BOC, UK)

Gas Regulator (BOC, UK)

Extractor Fan / filter Kit 2000 with hood and lamp (Nederman/ VWR International)

Low energy light bulb 20w (equivalent to 100w) fitted to lamp (Various)

Small tissue clips / green braided polyester suture 2 metric 3-0 UPS (FMI, Germany)

Calibration weight 5g (Ohaus).

Black thread (Various)

Krebs Gelatine Ringer solution

Gelatin / NaCl / KCl / KH_2PO_4 stock (4X store sterile at 4-8°C, warm to RT before use)

27.6g/L or 55.2g/2L NaCl

1.44g/L or 2.88g/2L KCl

0.64g/L or 1.28g/2L KH_2PO_4

8g/L or 16g/2L gelatin

Heat until all the gelatin is completely dissolved, adjust volume with ion-exchanged, distilled water to exactly 1 or 2L, mix, aliquot 4X 500ml and autoclave.

Sodium hydrogen carbonate stock / NaHCO_3 (833mM) – 21g per 300ml

Magnesium sulphate heptahydrate stock / $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (120mM) – 2.96g per 100ml

Calcium chloride 2-hydrate stock / $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (250mM) – 3.68g per 100ml

D-glucose / $\text{C}_6\text{H}_{12}\text{O}_6$ (2.22M) – 39.6g per 100ml

Store all stock solutions at 4-8°C.

Make up 1L of fresh Krebs solution daily from the stock solutions:

To exactly 250ml of the gelatin stock add approximately 600ml distilled water whilst mixing on a magnetic stirrer, then add 30ml of the Sodium hydrogen carbonate stock solution and 10ml of each of the Magnesium sulphate heptahydrate stock and the Calcium chloride 2-hydrate stock solutions and 5ml of the D-glucose stock (2.22 M) or 1.98g and adjust the pH to 7.5 if required and finally the volume to exactly 1L.

Krebs with the final composition should be produced:

Gelatine (0.2%), NaCl (118mM), KCl (4.83mM), KH_2PO_4 (1.19mM), NaHCO_3 (25mM), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1.2mM), D-glucose / $\text{C}_6\text{H}_{12}\text{O}_6$ (11.1 mM), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (2.54mM).

This solution is continuously oxygenated with carbogen (95% Oxygen/5% Carbon dioxide) gas.

Method

(i) Turn on PowerLab - The left most indicator on the front panel, the Power indicator, should glow green. The second indicator from the left of the front panel, the Status indicator, should flash yellow 4 times (indicating a SCSI ID number of 4). The status indicator will then turn off (If not consult Powerlab manual). Turn on computer and monitor.

(ii) Access chart recorder

Double click the mouse on; 4 channels icon, Chart v5 should open. Settings saved should be: 400 samples/sec. High pass = DC, Low pass = 200Hz, Range = 1mV. Digital filter = Low pass / 40Hz Cut off frequency.

Data pad settings: Time, Comment text, Ch1 freq, Ch2 freq, Ch1 Avg, Ch2 Avg, Ch3 freq, Ch4 freq, Ch3 Avg, Ch4 Avg, Comment time.

(iii) Calibration

Click mouse on (set up) and go to (zero all inputs). Start chart. Using the 5g Ohaus calibration weight, attach for a short time to each transducer. Stop the trace. Select the area of trace for channel 1 to include the 5g weight area. Click on (channel 1) and go to (units conversion). Select area of the base line and click the boxed arrow pointing to zero units. Select area representing the 5g displacement and click on the boxed arrow pointing to 5 units. Click on (apply), then (O.K.)

Repeat the procedure for all remaining channels.

(iv) Dissection / set up.

Wrap an ice block with blue roll. Place some Krebs (gassed with 95% O₂ / 5% CO₂) on a separate ice block. Obtain 20-30g (male) MF1 mice from the B.S.S. (order at ~ 20-25g). Transfer the mice in a spare cage to the dissection area. Kill the mice by cervical dislocation (A schedule 1 method). Avoid over stretching the mice as this may damage the diaphragms neuromuscular junction / nerve or sever the phrenic nerves. Place the mice under the lamp/extractor hood on the ice block.

Lay the animal on its back and remove the fur and skin covering the upper part of the abdomen and thorax. Remove the muscle layers covering the chest and expose the rib

cage. Lift the rib cage along the midline with a pair of forceps and make an incision half way between the xiphisternum and the neck. Examine inside the thoracic cavity to ensure that the phrenic nerves are not adhering to the chest wall as this occasionally happens. If this is the case, gently dislodge the nerves. Make lateral cuts on either side of the midline incision, parallel to the ribs, and fill the cavity with cold gassed Krebs solution. Lift the caudal edge of the incised rib cage and remove all the ribs except the one attached to the diaphragm on the animals left side. Carefully free the left phrenic nerve of connective tissue / fat and tie a short length of dark coloured thread around the uppermost part of the nerve and cut the nerve above the attached thread. Cut the wall of the abdomen to free the rib cage. The diaphragm and phrenic nerve should be irrigated regularly with cold gassed Krebs. Holding the xiphisternum with forceps cut straight down through the diaphragm to the spine, taking care not to cut or stretch the phrenic nerve. Cut the diaphragm laterally from its attachment to the body wall so freeing the preparation from the animal. Place the nerve and muscle in a petri dish containing gassed Krebs and trim the ribs / diaphragm to a suitable size to fit in the tissue bath. Attach a small tissue clip with an attached loop of thread to the tip of the tendonous tissue at the apex of the diaphragm preparation for later attachment to the transducer.

Quickly but gently locate the centre of the rib on the platinum spike electrode of the tissue holder and impale to secure the preparation. Gently pull the thread attached to the nerve through the ring electrode. Hook the loop from the tissue clip / diaphragm onto the force transducer and lower the mounted preparation into the organ bath containing gassed warmed Krebs. Adjust the tension to give a baseline tension of 1 - 1.5g (15mN). Gently pull the thread attached to the nerve until the tip of nerve is pulled through the ring electrodes. The nerve may be held in place by either suspending a small weight or attaching the thread with Blue tack. Start recording and stimulation of the nerve (~3V, 1Hz, 0.2ms). Regularly adjust the baseline tension to ~ 1.5g (15mN) as required. Ensure that the preparation is adequately aerated, but over aeration will produce excessive frothing. Nerve stimulation should be maintained throughout the experiment, except during washing or direct stimulation. Tissue preparations are stimulated indirectly (via the nerve) using a supramaximal voltage

(~3V). A short burst of direct (muscle) stimulation (~50V, 1Hz, lms) may also help the tissue to stabilise.

Wash the preparation with ~6ml fresh Krebs/gelatine at least twice. Adjust the tension as required until the baseline tension is consistent and no longer needs adjustment. Once a consistent twitch and baseline tension are produced without change over a period of greater than 30min the tissue is ready for toxin addition. No more adjustments should be made to the baseline tension for the rest of the experiment. If the twitch response is decreasing after this time or the preparation is producing responses of less than 0.5 gram tension then the preparation should either be given longer to stabilise or be discarded. The twitch response should be regular and consistent, if not the tissue should again either be given longer to stabilise or be discarded. No visible change to the baseline tension or twitch size should be found 30min prior to adding toxin.

Tissue baths are maintained at 37°C and contain 6ml of Krebs / gelatine oxygenated with 95% O₂ + 5% CO₂. A short burst of direct (muscle) stimulation (~50V, 1Hz, lms) is applied shortly (5-10min) before toxin addition and at the end of the experiment as an added control.

(v) Experimental protocol

Reconstitute toxin vials (therapeutic or reference preparations) typically with 1ml of gassed Krebs / gelatine. Dilute to the desired concentration (typically between 1 – 20 LD₅₀ U/ml) to give a total volume of 6ml Krebs / gelatine in a labelled bijou, mix gently and incubate at 37°C for 30 min. Stop the chart and drain the bathing Krebs solution, quickly but carefully add the toxin solution (pouring the toxin solution down the side of the tissue bath and not directly onto the tissue or thread connected with the force transducer) and immediately start the chart (Stimulation is automatically stopped when controlled by the PowerLab if the chart is not recording). Appropriately label the toxin addition on the chart. Click on the *, located at the bottom left of the chart, if a single channel is to be labelled then type the channel number and click add. If all channels are to be labelled then enter * and label the chart appropriately and click on add.

Typically after up to three and a half hours after adding the toxin, directly (muscle) stimulate the tissue and stop the chart. Click the mouse onto the (save) icon and appropriately label the file and save.

Discard or remove tissue and store for further studies. Discard remaining Krebs. Lower tissue holders back into the tissue baths and wash out thoroughly with tap water followed by distilled water. Once every month or as necessary clean the system with dilute (10%) sodium hypochlorite solution. Ensure all hypochlorite is thoroughly washed out of the system with several washes of distilled water and spills have been thoroughly washed off (as it will kill the tissue and is highly corrosive). The gas should not be turned off during cleaning or washing to prevent the back flow of liquid into the gas pipe. Tissue baths and tissue holders should be bathed and washed with fresh Krebs/gelatine thoroughly before further use.

Antibody neutralisation of toxin is assessed by premixing a fixed toxin concentration with the antitoxin and incubating for 30 minutes at 37°C before addition to the tissue at T0. An identical dose cycle is used to that shown above.

(vi) Data analysis:

Measure the average twitch height (10-20 twitches) before toxin addition and at 10 min intervals following toxin addition. Express as a percentage of twitch height before toxin addition. Plot the percentage twitch height against time and calculate the time to 50% inhibition.

Following toxin addition the twitch size should vary by less than 10% over the first 30 min. Any data falling outside these limits should be either excluded or if the decrease occurs in the first 10 min due to tissue movement but then remains stable, the data may be recalculated with 100% contraction set at 30min post toxin addition. (It is, however, preferable to have 5% or less deviation if possible.).

Using a reference toxin preparation with a known potency perform a dose response curve using at least 4-6 replicates per dose. Unknown toxin concentrations can then be calculated by repeatedly testing (at least 4 times) at a suitable dilution which can be

read off the linear part of the dose response curve. Other suitable methods of calculating the relative potency such as parallel line analysis may also be considered.

NATIONAL INSTITUTE FOR BIOLOGICAL STANDARDS AND CONTROL

Division of Bacteriology

Standard Operating Procedure

***In vitro* SNAP-25 endopeptidase Immunoassay for potency testing of
botulinum type A toxin preparations**

Written by: Y Liu

Authorised by: D Sesardic

INTRODUCTION

The L-chain of Botulinum neurotoxin type-A is known to contain a zinc-dependent endopeptidase enzyme whose toxic action results from cleavage of the critical synaptic protein SNAP-25. The assay is based on the immunochemical estimation of the cleavage of the SNAP-25 substrate by BoNT/A. A fragment of SNAP-25 spanning the toxin cleavage site (137-206) is immobilized onto wells of a microtitre plate and subsequently treated with BoNT/A. This results in a new epitope (SNAP-25₁₉₀₋₁₉₇) being exposed which was previously concealed. This new epitope is then measured with a specific antibody (anti-SNAP-25₁₉₀₋₁₉₇) to obtain an estimate of toxin endopeptidase activity.

MATERIALS AND REAGENTS

Materials

NUNC Maxisorb ELISA plates
Graduated pipettes (25ml, 10ml, 5ml)
Multichannel pipette and tips
Gilson (P1000, P200, P100, P20) (in calibration)
Measuring cylinder (1L)
Multiscan ELISA plate reader running Genesis software
pH meter (in calibration)
Electronic balance (in calibration)

Reagents

Coating Buffer (0.1M NaHCO₃/CO₃, pH 9.6)
Phosphate Buffer Saline (PBS)
PBS + 0.05% Tween-20 (PBST)
Marvel (skimmed milk powder)
HEPES (Sigma, H-3375, FW: 238.3)
Bovine Serum Albumin (BSA, Sigma, A0281, fatty acid free, globulin free)
DL-Dithiothreitol (DTT, Sigma, D5545)
Synthetic peptide substrate SNAP-25₁₃₇₋₂₀₆

Toxin:

Botulinum Toxin A (in house product specific reference, product batch)

Pre-reduction buffer: 50mM HEPES-NaOH, pH 7.0, + 20μM ZnCl₂, + 10mM DTT

Reaction Buffer: 50mM HEPES-NaOH, pH 7.0, + 20μM ZnCl₂, + 5mM DTT + 1mg/ml BSA

Substrate: Synthetic SNAP-25 peptide (SNAP-25₁₃₇₋₂₀₆):

70aa substrate (GGFIRRVTDN ARENEMDENL EQVSGIIGNL RHMALDMGNE IDTQNRQIDR IMEKADSNKT RIDEANQRAT KMLGSG) [Ekong *et al*, 1997]. Synthesized and purified (>80% pure).

Stock solution @10mg/ml aliquoted and stored at -20C.

Detecting antibody: Primary Antibody: Site-specific anti-peptide (CTRIDEANQ) antibody raised in New Zealand White rabbits. Specificity as reported in Ekong *et al*, 1997, Ekong *et al*, 1995.

Secondary Antibody: Goat anti-rabbit HRP conjugate (Sigma, A0545)

Substrate: 2, 2'-Azino-bis (3-ethylbenzthiozoline 6 sulfonic acid) (ABST) (Sigma, A9941)

Calibration checks and equipment checks related to this SOP

Before commencement of assay the following equipment calibrations must be carried out:

1. Calibrations of balance according to Bacteriology (NIBSC SOP QM005)
2. Calibrations of pH meter to pH 4 and 7
3. Calibrations of Gilson pipettes annually by qualified technician and a four monthly validation check laboratory personnel (NIBSC SOP PIP)

Unless otherwise stated in the SOP there is no requirement to use volumetric glassware in traceable calibration of the preparation of reagents, solutions or dilutions used in this SOP. Semi-automated pipettes, disposable plastic graduated pipettes, syringes, measuring cylinders and glassware are appropriate to the volumes being used, are adequate for this purpose. Volumes less than 1 ml are dispensed using Gilson pipettes in calibration. All reagents used in the preparation of solutions should be General Purpose Reagent grade, unless otherwise stated.

PROCEDURE FOR ENDOPEPTIDASE ASSAY

Immobilisation of SNAP-25 substrate

1. Prepare solution of 2µg/ml synthetic SNAP-25 substrate in coating buffer.
2. Add 100µl/well of SNAP-25 substrate solution to 96-well plates
3. Incubate at 4°C overnight.
4. Next day, wash plates 3x in PBST.
5. Add 150µl/well of 5% Marvel in PBST (M-PBST) to block.
6. Incubate for 1h at 37°C in a humidified box
7. Wash plates 3x with dH₂O and blot dry for immediate use

N.B Sealed plates can be stored at -20°C for up to 2 months.

Treatment of immobilised substrate with toxin

1. For toxin samples with 100U/vial: 8 vials of toxin reference, 6 vials of toxin test sample are needed
For toxin samples with 500U/vial: 4 vials of toxin reference, 3 vials of toxin test sample are needed
(NB. 2 Samples can be tested in 1 assay)

2. Label toxin sample vials as follows:
S1, S2, S3
T1, T2, T3 (if more than 1 sample is to be assay)
Label product specific reference vials as R1, R2, R3, R4, A (plate control and main reference)
3. Reconstitute test sample(s) and product specific reference toxin sample in Pre-reduction buffer as follows:
Carefully remove caps and stoppers ensuring that none of the material is lost.

Sample A (100U/vial): Add 100µl/vial of Pre-reduction buffer.
Pool vials of the same preparation to give a total of 3 samples vials and 4 reference vials.

Sample B (500U/vial): Add 300µl/vial of Pre-reduction buffer

4. Gently mix to ensure all the material is dissolved and incubate vials for 20mins at 37°C.
5. Place SNAP-25 coated plates on ice. (if plates have been stored, wash plates 3x in dH₂O and blot dry prior to placing on ice)
6. Remove vials from incubator and place on ice.
7. Add 180µl/well of Reaction buffer to row A
Add 100µl/well of Reaction buffer to the remaining wells (rows B-H)
8. Add 20µl reduced toxin per well to Row A according to the plate layouts show below.

The plate layout is designed to minimize the edge effect and the main reference (A) is located in the centre of the plate to avoid any variations in the absorbance.

	1	2	3	4	5	6	7	8	9	10	11	12
PLATE 1	S1	S1	R1	R1	T1	T1	A	A	T2	T2	S2	S2
PLATE 2	T2	T2	S2	S2	R2	R2	A	A	T3	T3	S3	S3
PLATE 3	T3	T3	S3	S3	T1	T1	A	A	R3	R3	S1	S1
PLATE 4	T2	T2	S2	S2	R2	R2	A	A	R3	R3	S1	S1

9. Perform doubling dilutions down each plates straight after adding sample/reference to row A
10. Seal the plates individually with self-adhesive tape and incubate plates in a humidified container for 60mins at 37°C (do not stack).

Estimation of immobilized intact and cleaved SNAP-25 substrate

1. Wash toxin treated plates 3x in PBST. Blot dry.
2. Add 100µl/well of 5µg/ml R-14 in 2.5% M-PBST (Primary-Ab)
3. Incubate for 90mins at 37°C
4. Wash plates 3x in PBST. Blot dry.
5. Add 100µl/well of 1/2000 goat anti-rabbit-HRP conjugate in 2.5% M-PBST
6. Incubate for 90mins at 37°C
7. Add 100µl/well ABTS substrate solution
8. Allow colour to develop at room temperature (leave for ~30-45mins)
9. Shake and read absorbance at 405nm using the ELISA plate reader.

Statistical Analysis

1. Absorbance reading obtained from Multiscan plate reader is transferred to word document with the appropriate plate layout and dilutions.
2. Using the bioassay program RANDOM, three points are chosen that are linear and parallel to the plate control reference (reference). The potency of the test samples is calculated relative to the main reference (A) by multiply the potency value of the test sample (not the log form) by the assigned unit/vial value of the main reference. Thus expressing the potency relative to the reference.
3. The potency values of the test samples is entered into Excel work sheet to calculate the overall mean of the test samples (\pm S.D) [see example]
4. The mean potency value of the test samples obtained from the *in vitro* endopeptidase assay needs to fall within the Manufacturer's Specifications and limits (\pm 15%) or the European Pharmacopoeia specifications and limits (\pm 20%) in order to be released by NIBSC.

If test sample fails to meet either specification the sample is assayed *in vivo* using the mouse local flaccid paralysis assay.

Example:

Layout

	1	2	3	4	5	6	7	8	9	10	11	12
Plate1	S1	S1	R1	R1	T1	T1	A	A	T2	T2	S2	S2
Plate2	T2	T2	S2	S2	R2	R2	A	A	T3	T3	S3	S3
Plate3	T3	T3	S3	S3	T1	T1	A	A	R3	R3	S1	S1
Plate4	T2	T2	S2	S2	R2	R2	A	A	R3	R3	S1	S1

Vial	Replicate 1	Replicate 2	Replicate 3	mean	plate control (REF)
S1	711	598	532	613.7	650
S2	732	499	433	554.7	605
S3	502	515		508.5	597
					537
					593
					596.4 40.27
		Overall mean (S.D.) of # Test			
		Sample =		558.9 52.71	

BUFFERS FOR ELISA

A. Phosphate Buffered Saline pH.7.4 (20x)

CHEMICAL	AMOUNT
Sodium chloride	800g
Potassium dihydrogen orthophosphate	20g
Di-Na-H-orthophosphate (dihydrate)	143g
Potassium chloride	20g
Distilled Water	Made up to 5 Litres

B. PBS/0.05% Tween

CHEMICALS	VOLUME
20x PBS solution	250ml
Tween 20	2.5ml
Distilled Water	Made up to 5 Litres

C. 0.05M Citric Acid, pH 4.0 (ABTS Substrate buffer)

CHEMICALS	AMOUNT	AMOUNT (2.5L)
Citrate monohydrate	10.51 g	26.28g
Distilled Water	Made up to 1 Litre	Made up to 2.5 Litres

D. Carbonate buffer, pH 9.6 (ELISA plate coating buffer)

CHEMICALS	VOLUME	VOLUME (2.5L)
Sodium carbonate, 0.015M	1.59g	3.98g
Sodium Hydrogen carbonate, 0.035M	2.93g	7.33
Distilled Water	Made up to 1 Litre	Made up to 2.5 Litres

- Store at 4°C for up to 2 weeks. For long term storage, autoclave (121°C for 15mins), store up to at 4°C

E. 50mM HEPES-NaOH buffer, pH 7.0 + 20µM ZnCl₂

CHEMICALS	AMOUNT	AMOUNT (2.5L)
50mM HEPES	2 litre	2.5 Litres
10mM ZnCl ₂	4ml	5ml

- 50mM HEPES: 11.915g in 1 litre dH₂O
- 10mM ZnCl₂ (FW: 136.3): 0.0136g in 10ml dH₂O
- Dilute ZnCl₂ to 20µM in HEPES buffer (1/500 dil). Adjust pH to 7.0 using 10M NaOH and Store at 4°C.

NATIONAL INSTITUTE FOR BIOLOGICAL STANDARDS AND CONTROL

Division of Bacteriology

Standard Operating Procedure

Non lethal mouse local muscular paralysis assay:

***In vivo* assessment of botulinum type A toxin**

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Authorized by: Dr D Sesardic

Introduction

Botulinum toxin type A induces muscular paralysis following specific binding and uptake by the pre-synaptic nerve terminal, and subsequent cleavage of SNAP25, a protein essential for the release of neurotransmitter at the synaptic junction. The non-lethal muscular paralysis assay relies on the measurement of the local flaccid muscular paralysis at the inguino-crural region of mice injected with a low dose of botulinum toxin (Takahashi *et al.* 1990; Sesardic *et al.* 1996). Toxin potency is reflected by the degree of muscular paralysis induced by the toxin. Activity of a toxin batch is estimated relative to a product specific toxin reference material of defined activity. The method is regulated under the UK Animal (Scientific procedures Act) 1986. It is the responsibility of the operator to ensure that the procedures they carry out are performed strictly in accordance with the method stated on the project licence and that their personal licence covers the techniques they are going to undertake.

Equipment:

class II EPC safety cabinet
sterile syringes 1ml
sterile needles 27G x 0.5" (0.4 x 12mm), and 21G x 1.5" (0.8 x 40mm)
pH meter
heater / stirrer
electronic balance
racks for tubes

Reagents and Chemicals:

Gelatin Phosphate Buffer, pH 6.5 (GPB)	
0.2% Gelatin	2g
0.05M Disodium hydrogen orthophosphate (2 hydrate)	8.9g
Water (Milli Q)	up to 1L

- Warm and stir the mixture until all the solids have dissolved.
- Allow to cool to room temperature and adjust the pH to 6.5 with 50% orthophosphoric acid and make up to 1L.
- Decant solution into 150ml glass bottles and sterilize by autoclaving at 121°C for 25 minutes. Store solution at +4°C.

Sample storage

Samples of botulinum type A toxin are stored according to the manufacturer's instructions. E.g. either in the fridge 4 to 8°C or freezer -15 to -45°C, as appropriate.

Animals:

Female mice, strain MF1 (Harlan) weighing 17-22g, are allowed to acclimatise according to in house standard procedures. Groups of 4 mice per cage are used for testing and each mouse is colour coded for identification. Animals are housed as indicated in the in house SOP for animal husbandry with free access to food and water.

Procedure:

Note – all work should be done in a class II EPC safety cabinet or a clean working area as appropriate.

Preparation of dilutions:

Using a 1ml syringe and needle (21G x 1.5") vials are carefully reconstituted with 1ml of GPB (Add GPB so that it strikes the wall of the vial first, to avoid vigorous agitation of the toxin) to give nominal potencies of 500U/ml assuming 500U/vial or 100U/ml assuming 100U/vial. Mix gently making sure that all the powder is dissolved and allow to stand at room for approximately 10 minutes.

Combine contents of duplicate vials using a fresh syringe and needle or after removing the vial stoppers and mix and dilute to 50U/ml (e.g. 100µl of 500U/ml nominal potency + 900µl GPB or 200µl of 100U/ml nominal potency + 200µl GPB).

A suitable range of dilutions should be made, e.g.:

Concentration U/ml	Amount (ii) toxin µl	Amount GPB µl	Sample
2.1	210	4790	REF, sample 1 (and 2)
1.5	150	4850	REF, sample 1 (and 2)
1.0	100	4900	REF, sample 1 (and 2)
0.5	50	4950	REF, sample 1 (and 2)

or

Concentration U/ml	Amount (ii) toxin µl	Amount GPB µl	Sample
1.5	150	4850	REF, sample 1 (and 2)
0.8	80	4920	REF, sample 1 (and 2)
0.4	40	4960	REF, sample 1 (and 2)
0.25	25	4975	REF, sample 1 (and 2)

Syringes (1ml with 27G x 0.5") are numbered and samples randomised before injection in two blocks of cages so that each dose of each preparation occurs once in each of the two blocks. Randomisation is carried out for each assay, and two examples of this randomization are shown below using one or two test samples.

Cage / syringe number	Sample	Dose	Number of Mice
1	-ve control GPB	0	4 per cage
2	REF	2.1 U/ml	4 per cage
6	REF	1.5 U/ml	4 per cage
9	REF	1 U/ml	4 per cage
7	REF	0.5 U/ml	4 per cage
4	Test Sample 1	2.1 U/ml	4 per cage
8	Test Sample 1	1.5 U/ml	4 per cage
5	Test Sample 1	1 U/ml	4 per cage
3	Test Sample 1	0.5 U/ml	4 per cage
17	REF	2.1 U/ml	4 per cage
12	REF	1.5 U/ml	4 per cage
16	REF	1 U/ml	4 per cage
14	REF	0.5 U/ml	4 per cage
10	Test Sample 1	2.1 U/ml	4 per cage
11	Test Sample 1	1.5 U/ml	4 per cage
13	Test Sample 1	1 U/ml	4 per cage
15	Test Sample 1	0.5 U/ml	4 per cage

Cage / syringe number	Sample	Dose	Number of Mice
1	-ve control GPB	0	4 per cage
2	REF	2.1 U/ml	4 per cage
12	REF	1.5 U/ml	4 per cage
6	REF	1 U/ml	4 per cage
7	REF	0.5 U/ml	4 per cage
4	Test Sample 1	2.1 U/ml	4 per cage
11	Test Sample 1	1.5 U/ml	4 per cage
5	Test Sample 1	1 U/ml	4 per cage
13	Test Sample 1	0.5 U/ml	4 per cage
3	Test Sample 2	2.1 U/ml	4 per cage
8	Test Sample 2	1.5 U/ml	4 per cage
9	Test Sample 2	1 U/ml	4 per cage
10	Test Sample 2	0.5 U/ml	4 per cage
17	REF	2.1 U/ml	4 per cage
25	REF	1.5 U/ml	4 per cage
20	REF	1 U/ml	4 per cage
21	REF	0.5 U/ml	4 per cage
18	Test Sample 1	2.1 U/ml	4 per cage
19	Test Sample 1	1.5 U/ml	4 per cage
22	Test Sample 1	1 U/ml	4 per cage
15	Test Sample 1	0.5 U/ml	4 per cage
24	Test Sample 2	2.1 U/ml	4 per cage
16	Test Sample 2	1.5 U/ml	4 per cage
14	Test Sample 2	1 U/ml	4 per cage
23	Test Sample 2	0.5 U/ml	4 per cage

Dosing and monitoring of animals:

Eight mice (2 cages of 4) receive an injection (s.c.) of 0.1ml volume of each dilution in the left inguinocrural region. Two operators are required to administer the injections, one person to hold the animal while the other carefully injects 0.1ml taking care not to inject too deeply.

Following injection of botulinum toxin, the animals are checked for signs of muscular paralysis at the inguinocrural / abdominal region at 24h and 48h post-injection (sometimes at 30h if required). Any animals showing slight signs of systemic toxicity should be more regularly monitored and culled immediately if moderate or severe signs of toxicity develop.

Scoring:

Scoring should be independently performed by trained individuals, preferably blind to the randomisation. Colour coded mice are picked up individually by their tail and scored independently by each observer.

0 : No signs, normal

1: Just detectable (slight bulge at injection site). E.g. Covering an area of approximately 0.5cm diameter or less (or less than two nipples).

2 : More pronounced bulge. E.g. Covering an area of greater than 0.5cm diameter (or greater than or equal to two nipples), but less than the maximum radius of the hind leg heal.

3: More extensive bulge extending over a larger area. Extending below hips / top of thigh when viewed from the side and beyond the maximum radius of the hind leg heal.

4: Maximal local effect. More extensive bulge extending over a larger area will often extend as far as the bottom of the rib cage, or over a large area with extensive distension or bulging.

‘Beyond a 4’.: When high concentrations of toxin are used a No4 bulge may flatten out with time (e.g. at 48h) or go ‘beyond a 4’. This may be an early (e.g. 24h) indicator of subsequent systemic signs of toxicity.

Examples of scoring forms are shown below. Additional sheets are used if additional times or other characteristics of the mice, such as weight, are recorded.

Title: Botulinum toxin (type A) Non lethal assay (PPL 80/_____) **Date of assay:** _____. **Test ID number:** _____

M1=Blue head, M2= Blue tail, M3=Pink head, M4=Pink tail.

Time Scored: _____ **Observer Initials:** _____

Time	Cage	M1 Blue H	M2 Blue T	M3 Pink H	M4 Pink T	Total score	Comment
24hr	1						
	2						
	3						
	4						
	5						
	6						
	7						
	8						
	9						
	10						
	11						
	12						
	13						
	14						
	15						
	16						
	17						

Time Scored: _____ Observer Initials: _____

Time	Cage	M1 Blue H	M2 Blue T	M3 Pink H	M4 Pink T	Total score	Comment
48hr	1						
	2						
	3						
	4						
	5						
	6						
	7						
	8						
	9						
	10						
	11						
	12						
	13						
	14						
	15						
	16						
	17						

Data analysis for the consistency of scorers:

The scores from independent inspectors for each dilution are collected. Consistency between inspectors is assessed. If less than 60% of mice are scored identically and / or more than 4% of the mice have a score difference of 2 or more then the data should be referred to a statistician for a more detailed consideration of the differences between inspectors.

Data analysis for potency:

Reference concentrations should be adjusted for any difference between the actual assigned value and the assumed value of 500 or 100 U/vial (e.g. Actual assigned value divided by assumed value of 500 or 100U X Assumed dilution concentration).

The mean score for each cage is calculated and dose response curves plotted. Activity of the test sample is estimated by comparing with the response obtained with the reference standard. Using the linear region of the dose response curve, a parallel line analysis is performed and potency of test sample calculated relative to the reference standard. Product specific standard of defined activity is included in each assay.